

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problems Mailbox.**



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 48/00	A1	(11) International Publication Number: WO 98/09656 (43) International Publication Date: 12 March 1998 (12.03.98)
(21) International Application Number: PCT/US97/15690 (22) International Filing Date: 4 September 1997 (04.09.97) (30) Priority Data: 60/025,549 6 September 1996 (06.09.96) US 08/889,930 10 July 1997 (10.07.97) US (60) Parent Applications or Grants (63) Related by Continuation US 60/025,549 (CON) Filed on 6 September 1996 (06.09.96) UZ 08/889,930 (CON) Filed on 10 July 1997 (10.07.97) (71) Applicant (for all designated States except US): TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; Suite 300, 3700 Market Street, Philadelphia, PA 19104-3147 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): WILSON, James, M. [US/US]; 1350 N. Avignon Drive, Gladwyne, PA 19035 (US). CHEN, Youhai [CA/US]; 205 Cherry Hill Lane, Broomall, PA 19008 (US).		(74) Agents: BAK, Mary, E. et al.; Howson and Howson, Spring House Corporate Center, P.O. Box 457, Spring House, PA 19477 (US). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: METHOD FOR TOLERIZING A MAMMALIAN PATIENT TO ADMINISTRATION OF GENE THERAPY VIRUS VECTORS (57) Abstract <p>A method for tolerizing a mammalian subject to administration of a live virus carrying a gene for delivery to a cell of the subject is disclosed. The method entails administering to the subject a suitable amount of an inactivated virus prior to administration of the live virus. The prior administration of the inactivated virus suppresses anti-virus cytotoxic T cells, permitting longer transgene persistence once the live virus is administered, and permitting effective readministration of live virus.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

METHOD FOR TOLERIZING A MAMMALIAN PATIENT TO
ADMINISTRATION OF GENE THERAPY VIRUS VECTORS

This invention was supported by the National
Institute of Health Grant No. DK47757. The United States
5 government has rights in this invention.

Field of the Invention

The present invention relates to the field of
somatic gene therapy and methods useful therein.

Background of the Invention

10 Immune responses of the recipient to the viral
vectors used in somatic gene therapy, i.e., to the viral
proteins of the vector, and/or to the transgene carried
by the vector, and/or the virus-infected cells, have
emerged as recurring problems in the initial application
15 of gene therapy technology to animals and humans [Yang et
al, J. Virol., 69:2004-2015 (1995) (Yang I)]. In
virtually all models, including lung-directed and liver-
directed gene therapy, expression of the transgene is
transient and associated with the development of
20 pathology at the site of gene transfer.

The transient nature of transgene expression from
recombinant adenoviruses, for example, has been found to
be due, in part, to the development of antigen specific
cellular immune responses to the virus-infected cells and
25 their subsequent elimination by the host. The
collaboration of CTLs directed against newly synthesized
viral proteins and viral specific T helper cells [Zabner
et al, Cell, 75:207-216 (1993); Crystal et al, Nat.
Genet., 8:42-51 (1994)] leads to the destruction of the
30 virus-infected cells. These immune responses have also
been noted to cause the occurrence of associated
hepatitis that develops in recipients of *in vivo* liver-
directed gen therapy within 2-3 weeks of initial

treatment, and myositis in recipients of *in vivo* muscle-directed gene therapy.

Another antigenic target for immune mediated clearance of virus-infected cells is the product of the transgene. CTLs are an important effector in the destruction of target cells with activation occurring in some cases in the context of the transgene product, as well as the viral-synthesized proteins, both presented by MHC class I molecules [Yang I; and Zsengeller et al, Hum. Gene Thera., 6:457-467 (1995)].

Another limitation of the use of recombinant virus vectors for gene therapy has been the difficulty in obtaining detectable gene transfer upon a second administration of virus. This limitation is particularly problematic in the treatment of single gene inherited disorders or chronic diseases, such as cystic fibrosis (CF), that will require repeated therapies to obtain life-long genetic reconstitution. Diminished gene transfer following a second therapy has been demonstrated in a wide variety of animal models following intravenous or intratracheal delivery of adenovirus vectors [T. Smith et al, Gene Thera., 5:397 (1993); S. Yei et al, Gene Thera., 1:192-200 (1994); K. Kozarsky et al, J. Biol. Chem., 269:13695 (1994)]. Similar difficulties have been noted when the viral vector is other than adenovirus, i.e., retrovirus, vaccinia, and the like. In each case, resistance to repeated gene therapy was associated with the development of neutralizing anti-virus antibodies, which thwarted successful gene transfer following a second administration of virus.

Proposed solutions to these anti-viral immune responses have to date involved new designs of the virus vectors which employ fewer viral genes, as well as the pre- or co-administration of immune modulators, such as anti-CD40 ligands and other modulators identified in the

art [See, e.g., Yang et al., J. Virol., 70(9) (Sept., 1996); International Patent Application No. WO96/12406, published May 2, 1996; and International Patent Application No. PCT/US96/03035, all incorporated herein
5 by reference].

Nevertheless, the successful induction of specific immune tolerance to the new gene products (both virus products and transgene products) of the recombinant gene therapy vectors remains one of the most formidable
10 challenges in gene therapy. Failure to establish immune tolerance to viral gene therapy vectors may lead to immune rejection of the transgene-expressing cells and therefore loss of transgenes.

There exists a need in the art for methods and
15 compositions which enable the induction of specific immunologic tolerance to viral vector capsid proteins and both viral gene and transgene products being introduced and expressed in mammalian cells by general gene therapy methods.

20 Summary of the Invention

The present invention provides a solution to the aforementioned need in the art by providing a method for eliminating the immune barrier impeding long-term gene therapy by selectively tolerizing a mammalian subject to
25 multiple administrations of a live recombinant virus carrying a gene for delivery to a cell of said subject. This method involves administering to the subject a suitable amount of an inactivated virus prior to administration of a first live recombinant virus. The
30 inactivated virus may be a whole virus, a replication defective virus, a previously inactivated copy of the live recombinant virus carrying a transgene, or a previously inactivated recombinant virus without a transgene. Inactivated virus is preferably administered

via an oral or intravenous route. The administration of the inactivated virus essentially deactivates T cells specific for viral antigens, before the live recombinant virus carrying the transgene is administered.

- 5 Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

Brief Description of the Drawings

- Fig. 1A is a graphical plot of the data obtained from the proliferation assay described in Example 2. The graph plots viral proliferation in mouse splenocytes, measured by radioactivity of tritiated thymidine in units of CPM X 10^{-3} as a function of the number of recombinant adenovirus serotype 5 virus carrying a beta-galactosidase gene (rAd5-LacZ)/ml X 10^{-8} . C57BL/6 mice were fed with (1) PBS (open square), (2) 10^{12} heat-inactivated, UV-irradiated rAd5-LacZ ("dead" rAd5-LacZ) (open circle), or cells from mice injected intravenously (iv) with 10^{12} dead rAd5-LacZ (filled circle), and subsequently challenged with live rAd5-LacZ. Splenocytes were harvested for analysis 10 days after administration of live rAd5-LacZ.

- Fig. 1B is a graphical plot of the data obtained from the cytokine (IL-2) assay described in Example 2. The graph plots IL-2 in pg/ml as a function of number of rAd5-LacZ/ml X 10^{-8} for the same mice as reported in Fig. 1A.

- Fig. 1C is a graphical plot of the data obtained from the cytokine (IFN- γ) assay described in Example 2. The graph plots IFN- γ in pg/ml as a function of number of rAd5-LacZ/ml X 10^{-8} for the same mice as reported in Fig. 1A.

Fig. 1D is a graphical plot of the data obtained from the cytokine (IL-10) assay described in Example 2. The graph plots IL-10 in pg/ml as a function of number of rAd5-LacZ/ml $\times 10^{-8}$ for the same mice as reported in Fig. 1A.

Fig. 2 is a graphical plot of the data obtained from the cytotoxicity assay described in Example 3. The symbols report mice treated as described in Example 1. Open square, fed with PBS; open circle, fed with 10^{12} dead rAd5-LacZ; filled circle, injected intravenously with 10^{12} dead rAd5-LacZ. The graph plots % specific killing of splenocytes as a function of effector-to-target ratio.

Fig. 3A is a histogram of mouse lung tissue for mice fed with PBS and subsequently challenged with live rAd5-LacZ at day 10 following challenge. LacZ gene expression is observed.

Fig. 3B is a histogram of mouse lung tissue for mice fed with dead rAd5-LacZ and subsequently challenged with live rAd5-LacZ at day 10 following challenge. LacZ gene expression is observed.

Fig. 3C is a histogram of mouse lung tissue for mice fed with PBS and subsequently challenged with live rAd5-LacZ at day 17 following challenge. LacZ gene expression is observed.

Fig. 3D is a histogram of mouse lung tissue for mice fed with dead rAd5-LacZ and subsequently challenged with live rAd5-LacZ at day 17 following challenge. LacZ gene expression is observed.

Fig. 3E is a histogram of mouse lung tissue for mice fed with PBS and subsequently challenged with live rAd5-LacZ at day 28 following challenge. No LacZ gene expression is observed.

Fig. 3F is a histogram of mouse lung tissue for mice fed with dead rAd5-LacZ and subsequently challenged with live rAd5-LacZ at day 28 following challenge. LacZ gene expression is observed.

5 Detailed Description of the Invention

 The method of the present invention provides for selectively tolerizing a mammalian subject to multiple administrations of a recombinant gene therapy virus which carries a therapeutic gene (transgene) for delivery to a
10 cell, wherein the transgene product is expressed *in vivo*. According to the present invention, it has been observed unexpectedly that administering to a prospective candidate for gene therapy treatment a suitably high dosage of an inactivated virus prior to administration of
15 a live recombinant virus carrying a transgene suppresses the anti-virus immune responses mounted by the patient and permits prolonged transgene expression *in vivo*.

 As detailed in Example 1 below, oral administration of a heat-inactivated, UV-irradiated rAd5-LacZ induced
20 immune suppression of anti-adenoviral antigen immunity in mice that were subsequently challenged with live rAd5-LacZ. Immune tolerance was measured by determining the persistence of the transgene, LacZ, in the mouse tissue at various days following challenge. Mice receiving the
25 live rAd-LacZ did not reject the live recombinant virus carrying the transgene. In contrast, mice which received similar oral doses of live rAd5-LacZ or live rAd5-LacZ-infected splenocytes, displayed strong anti-adenoviral antigen immunity upon presentation with the live virus.
30 This result indicated that prior administration of the inactivated or killed virus induced immune tolerance in the mice for the administration of the live recombinant virus carrying the transgene.

Additional experiments described in detail in Example 2 below were conducted to determine if the immune tolerance to subsequent administration of the live recombinant virus resulting from oral administration of the inactivated virus could be similarly induced by iv administration of the inactivated virus. Following the same protocol as in Example 1, mice were administered phosphate buffered saline as a control or inactivated rAd5-LacZ orally or the same dosage of inactivated rAd5-LacZ i.v. Subsequently, all three groups of mice were challenged with live rAd-LacZ.

Mice splenocytes were subjected to a proliferation assay to measure immune tolerance based on a comparison of the number of splenocytes which proliferated as a function of the number of live rAd5-LacZ/ml. As illustrated in Fig. 1A, splenocytes decreased in number for the mice administered the inactivated virus, both orally and intravenously in comparison to the control.

Cytokine assays were conducted on the same mice to assess the mechanism of the tolerance. As shown in Fig. 1B IL-12 production was suppressed only with the orally administered inactivated virus. However, both IFN- γ (Fig. 1C) and IL-10 (Fig. 1D) were suppressed in response to administration of orally or i.v. administered inactivated virus in comparison to the control. Oral administration suppressed the two latter cytokines to a greater extent than both the control and the iv administered inactivated virus.

Because immune rejection of transgene-expressing cells are mediated primarily by cytotoxic T cells, anti-adenovirus cytotoxicity to oral or intravenous administration of dead viral antigens was tested by ^3H -thymidine release assay (JAM assay) as described in Example 3. As shown in Fig. 2, both oral and intravenous administrations of dead rAd5-LacZ suppressed generation

of adenovirus-specific cytotoxic T cells, although under these conditions, the oral route appeared to be more effective than the intravenous route. It is to be noted that this is the first demonstration that oral
5 administration of antigens activates anti-virus cytotoxic T cells.

Resistance to re-administration of recombinant virus may be partially mediated by neutralizing antibodies specific for viral antigens. Therefor to determine
10 whether oral administration of rAd5-LacZ suppresses humoral immunity, anti-adenovirus antibody responses in mice fed rAd5-LacZ were examined by conventional methods as described in Example 4 below. A 40-60% suppression was observed for anti-adenovirus IgM and IgG responses.
15 These data strongly suggest that specific immunologic tolerance can be induced by oral or intravenous administration of inactivated virus, but that oral route may be more effective than intravenous route.

To test whether oral tolerance helps to prolong the
20 *in vivo* transgene expression, LacZ gene expression was examined by X-gal histochemistry according to standard procedures as described in Example 5 for mice administered inactivated rAd5-LacZ or PBS orally and subsequently challenged with live recombinant virus as in
25 Example 2. As indicated in the Figs. 3A through 3F, LacZ gene expression lasted approximately 17 days in lung tissue of non-tolerized (PBS-fed) mice. The expression of the transgene was prolonged to at least 28 days in mice fed 10^{12} dead rAd5-LacZ prior to live virus
30 challenge, according to this invention.

Thus, pre-administration of inactivated virus has a 'tolerizing' effect upon subsequent administration of the live recombinant virus. This allows prolonged persistence of transgene expression from a single
35 administration of recombinant virus and allows effective

multiple administrations of a recombinant virus carrying the transgene to the gene therapy candidate, i.e., a patient suffering from a disorder due to the mutation, deletion or functional deletion of a normal gene or a patient with an acquired disease.

The method of the present invention is useful for any recombinant virus carrying a transgene useful in gene therapy, such as adenovirus, adeno-associated virus, retrovirus and others. Many suitable recombinant viruses vectors carrying a host of desired genes are presently known to the art. The design of the recombinant gene therapy virus is not a limitation on this method, nor is the selection of the transgene and the regulatory sequences required to express the transgene product *in vivo*. The selection of the virus is well within the skill of the art of gene therapy.

Similarly, it is anticipated that the method of this invention will operate to suppress unwanted anti-virus and possibly, anti-transgene, immune responses regardless of the disease or disorder being treated. Thus, this method is expected to have broad applicability in the field of gene therapy.

In one embodiment of this invention, the inactivated virus and the live recombinant virus carrying the transgene have substantially, and preferably completely, identical viral capsid proteins and viral genes. For example, as described in the examples below, the "dead virus" is simply the same recombinant virus, except that it has been killed or inactivated. The subsequent live virus administration uses the same virus, which has not been inactivated.

Alternatively, the inactivated virus may be the same virus, but does not carry a transgene, i.e., an "empty" recombinant virus.

In still another embodiment, the inactivated virus and live virus may have antigenically related viral proteins. "Antigenically related" means that each virus activates an immune response capable of eliminating the other virus, because each virus expresses a gene product with at least one antigen present on the other gene product.

In still a further embodiment, the inactivated virus may be an inactivated whole virus, the genome of which provided the viral sequences of the recombinant transgene-carrying virus, or a replication defective virus comprising at least a portion of the genome from which the recombinant transgene-carrying virus was made. The replication-defective virus may or may not also carry the transgene.

Another embodiment of the method of this invention permits tolerization of a patient to the open reading frame of the therapeutic transgene. According to this method, the inactivated virus is a different virus or strain than the live virus vector, but carries and expresses the same transgene as does the live virus vector.

The inactivated virus may be prepared by conventional means. Preferably for heat inactivation, live virus is subjected to temperatures of at least about 56°C for about 30 minutes. Similarly, the UV irradiation necessary to inactivate a virus can range from exposures of between about 100 to about 4000 rads for an exposure time of about several seconds to several hours. Other means of inactivation, such as chemical inactivation with organic solvents, toxic bleaches, imines, formalin and the like can be employed and are well-known to the art. Combinations of these conventional mechanisms of viral inactivation may also be employed to produce an inactivated virus of this invention.

In the practice of this invention, it is preferred that a high dose of the inactivated virus be administered via the oral route, as demonstrated in the following examples and figures referred to herein. However,
5 another suitable route of administration of inactivated virus is intravenous (iv). A dose of the inactivated virus suitably high to induce tolerance is greater than about 10^{11} viral particles/kg patient body weight for either route of administration. More preferably, where
10 the route is oral, a preferred amount of the inactivated virus is greater than 10^{13} particles/kg, and may be about, or greater than, 10^{16} particles/kg. Where the route is i.v., a preferred amount of the inactivated virus is greater than 10^{11} particles/kg, and may be
15 about, or greater than, 10^{14} particles/kg.

The amount of a particular inactivated virus necessary for a particular patient according to the methods of this invention may be determined on an empirical basis by one of skill in the art. Such an
20 amount may also be impacted by the specific virus employed (i.e., the degree to which it expresses viral antigens), and can be determined readily without resort to undue experimentation.

The inactivated virus is generally administered
25 between about 6 hours to about 28 days before administration of the first live recombinant virus carrying the transgene to permit the immune suppression or T cell de-activation to occur before administration of the live recombinant virus. Pre-administration of
30 inactivated virus creates a climate of immune tolerance, or at least reduced immunologic rejection, of the live recombinant virus.

Although it is not anticipated to be necessary, administration of the inactivated virus may be repeated
35 prior to a second or subsequent administration of the

live virus, in conditions such as cystic fibrosis, in which repeated transgene administration is likely to be necessary for the treatment to be effective. However, it is anticipated that only a single administration of the
5 inactivated virus prior to the first administration of the live recombinant virus carrying the transgene will be necessary to induce long-lasting tolerance.

Immune tolerance can be mediated by several different mechanisms which include deletion, anergy and
10 deviation of specific lymphocytes. Several mechanisms of immune tolerance are anticipated to be involved in this gene therapy method. While not wishing to be bound by theory, the mechanism by which the present invention operates may be similar to "clonal anergy", i.e., T cell
15 non-responsiveness, causing deactivation of immune attack T cells specific to a particular antigen, resulting in a reduction in the immune response to this antigen. See, e.g., the description in International Patent Application No. WO95/27500, published October 19, 1995 and
20 incorporated herein by reference.

It is anticipated that oral tolerance to the recombinant virus may be enhanced by manipulation of antigen dosage as described above. Other factors may also enhance oral tolerance, such as the strength of
25 costimulation and cytokine milieu. For example, oral tolerance may be enhanced by administering the inactivated virus with a selected immune modulator. An optional step in the method of this invention involves the co-administration to the patient, either concurrently with,
30 or before or after administration of the inactivated virus, of a suitable amount of a short acting immune modulator. The selected immune modulator is defined herein as an agent capable of inhibiting the formation of neutralizing antibodies directed against the recombinant
35 virus of this invention or capable of inhibiting

cytolytic T lymphocyte (CTL) elimination of the virus. The immune modulator may interfere with the interactions between the T helper subsets (T_{H1} or T_{H2}) and B cells to inhibit neutralizing antibody formation. Alternatively, 5 the immune modulator may inhibit the interaction between T_{H1} cells and CTLs to reduce the occurrence of CTL elimination of the recombinant virus.

A variety of useful immune modulators and dosages for use of same are disclosed, for example, in Yang et 10 al., *J. Virol.*, 70(9) (Sept., 1996); International Patent Application No. WO96/12406, published May 2, 1996; and International Patent Application No. PCT/US96/03035, all incorporated herein by reference.

The following examples and analyses of the method of 15 this invention employ a cystic fibrosis (CF) gene therapy model, using a recombinant replication-defective human adenovirus type 5 (rAd5) as a vector and *Escherichia coli lacZ* as transgene. However, it should be clear to one of skill in the art that the methods of this invention have 20 broad application to other disorders, other recombinant viruses and inactivated viruses and other transgenes. The scope of this invention is thus not limited to the components of the following examples used to illustrate the invention.

25 EXAMPLE 1: ANTIGEN SELECTION BASED ON ABILITY TO INDUCE ORAL TOLERANCE

Since the form of an antigen is known to play a pivotal role in the development of oral immunity versus tolerance, three types of viral antigens were examined 30 for their ability to induce oral tolerance or immunity. These include a live recombinant adenovirus, serotype 5 (Ad5) carrying a gene encoding beta-galactosidase (LacZ). This recombinant virus is described in Y. Yang et al, *J. Virol.*, 69(4):2004-2015 (1995) as H5.010CMVLacZ. It is

based on an Ad5 sub360 backbone deleted of E1a and E1b (1.0 to 9.6 map units) as well as 400 bp of the E3 region. LacZ is expressed from a cytomegalovirus promoter. For ease of reference, this recombinant virus is referred to herein as "live rAd5-LacZ".

A second viral antigen is heat-inactivated, ultraviolet (UV)-irradiated rAd5-LacZ. The recombinant virus was heated for 0.5 hour at 56°C, followed by exposure to ultraviolet radiation of about 1000 rads for several minutes. The third antigen is live rAd5-LacZ-infected syngeneic splenocytes.

To determine whether these antigen preparations induce oral tolerance, C57BL/6 mice were first fed with 10^{12} virus particles of one of the above-described live or inactivated virus vectors, or with 10^7 splenocytes infected with 10^{12} live virus particles.

Two days later, the mice were challenged with 5×10^{10} particles of live rAd5-LacZ intratracheally. Mice were sacrificed 10 days after the challenge and their spleens harvested. The splenocytes were tested for anti-adenoviral immune responses in a conventional proliferation assay as described in Yang et al., J. Virol., 69:2004 (1995), incorporated herein by reference.

Surprisingly, oral administration of heat-inactivated, UV-irradiated rAd5-LacZ induced profound immune suppression (tolerance) of anti-adenovirus immunity. In contrast, oral administration of live rAd5-LacZ or live rAd5-LacZ-infected syngeneic splenocytes induced no tolerance.

EXAMPLE 2: TOLERIZATION OF ADENOVIRUS-SPECIFIC HELPER T CELLS.

A. Tolerance/Challenge Experiment

Three groups of C57BL/6 mice were fed either with phosphate buffered saline (PBS) or 10^{12} particles of

heat-inactivated, UV-irradiated rAd5-LacZ ("dead" virus). As a control, the third group of mice was injected intravenously with 10^{12} particles of dead rAd5-LacZ to determine whether tolerance could be induced via an intravenous route of administration. Two days later, mice were challenged with 5×10^{10} particles of live rAd5-LacZ intratracheally. Mice were sacrificed 10 days after the challenge and their spleens harvested.

The splenocytes were tested for anti-adenovirus immune responses in a proliferation assay and a cytokine assay described below. The experiment was repeated twice with similar results.

B. Proliferation Assay

A proliferation assay was conducted as described in Yang et al., J. Virol., 69:2004 (1995), incorporated by reference. Briefly described, splenocytes, 1×10^6 cells/well, were cultured in 0.2 ml serum-free medium containing various concentrations of dead rAd5-LacZ. $1 \mu\text{Ci}$ of ^3H -thymidine was added to each culture 72 hours after initiation of the culture and cells were harvested and radioactivity measured 16 hours later.

The results of the proliferation assay can be seen in Fig. 1A. Radioactivity is measured in counts per minute of the tritiated thymidine incorporated into the DNA. These data points are proportional to cell proliferation. Each data point represents a mean from a minimum of 3 mice. The standard deviations of CPM data are within 25% of the means.

As illustrated in Fig. 1A, both oral and intravenous administrations of dead rAd5-LacZ suppressed splenocyte proliferation when compared with PBS controls. Splenocytes decreased in number for the mice administered the inactivated virus, both orally and intravenously in comparison to the control.

C. Cytokine Assays

To assess the mechanisms behind the immune suppression and cell proliferation, cytokine assays were conducted as described in Chen et al., Science, 265:1237 (1994), incorporated by reference.

Briefly described, culture supernatants were collected at 40 hours and cytokine concentration was determined by ELISA. Each data point represents a mean from a minimum of 3 mice and the standard deviations of the cytokine data are within 20% of the means. The results of the cytokine assays can be seen for IL-2 in Fig. 1B, for IFN- γ in Fig. 1C, and for IL-10 in Fig. 1D.

Oral, but not intravenous, pre-administration of inactivated rAd5-LacZ suppressed IL-2 production. However, both IFN- γ (Fig. 1C) and IL-10 (Fig. 1D) were suppressed in response to administration of orally or i.v. administered inactivated virus in comparison to the control.

EXAMPLE 3: TOLERIZATION OF VIRUS-SPECIFIC CYTOTOXIC T CELLS.

To test whether oral or intravenous administration of dead viral antigens affects cytotoxic T cell responses, anti-adenovirus-induced cytotoxicity was tested by ^3H -thymidine release assay (JAM assay) as described in P. Metzinger, J. Immunol. Meth., 145:185 (1991), incorporated herein by reference.

Briefly described, mice were treated as in Example 2 above. Their splenocytes were cultured *in vitro* for four days with rAd5-LacZ and their specific cytotoxicity tested using a syngeneic fibroblast cell line infected with rAd5-LacZ. CTLs will lyse cells with the viral antigens displayed on their surfaces. The cells are loaded with radio-label and the release of label is proportional to cell lysis.

The results are shown in Fig. 2. Each data point represents a mean from a minimum of 3 mice. The standard deviations are with 15% of the means. The experiment was repeated twice with similar results.

- 5 Both oral and intravenous administrations of dead rAd5-LacZ suppressed the generation of adenovirus-specific cytotoxic T cells, although under these conditions, the oral route appeared to be more effective than the intravenous route.

10 EXAMPLE 4: EFFECT ON HUMORAL IMMUNITY

- To test whether oral administration of inactivated rAd5-LacZ suppresses humoral immunity, anti-adenovirus antibody responses in mice fed inactivated rAd5-LacZ were examined by conventional methods as described in Yang et al., J. Virol., 69:2004 (1995), incorporated by reference herein. Briefly described, mice were treated as described in Example 2 and the adenovirus-specific antibody isotype was determined in bronchial alveolar lavage (BAL) by using enzyme-linked immunosorbent assay (ELISA).
- 15
- 20

- The resulting data showed that although mucosal IgA was not drastically suppressed by oral administration of inactivated rAd5-LacZ, a 40-60% suppression was observed for anti-adenovirus IgM and IgG responses. These data strongly suggest that specific immunologic tolerance can be induced by oral or intravenous administration of inactivated virus and that oral route may be more effective than intravenous route.
- 25

EXAMPLE 5: X-GAL HISTOCHEMISTRY

- 30 LacZ gene expression was examined by X-gal histochemistry according to standard procedures [see, e.g., procedures described in J. Price et al, Proc. Natl. Acad. Sci., USA, 84:156-160 (1987)]. Mice were treated

as described in Example 2 and their lung tissue examined at various times after live virus challenge.

As indicated in the Figs. 3A through 3F, LacZ gene expression lasted approximately 17 days in lung tissue of
5 non-tolerized (PBS fed) mice. The expression of the transgene was prolonged to at least 28 days in mice fed 10^{12} dead rAd5-LacZ, according to this invention.

All references recited above are incorporated herein by reference. Numerous modifications and variations of
10 the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the compositions and processes of the present invention, such as selections of different
15 transgenes or selection or dosage of the vectors or immune modulators are believed to be within the scope of the claims appended hereto.

WHAT IS CLAIMED IS:

1. Use of an inactivated virus in preparing a medicament for administration to a mammalian subject, wherein said inactivated virus is administered in a high dose prior to administration of a live recombinant virus carrying a transgene for delivery to said subject, wherein said medicament tolerizes said mammalian subject to said live recombinant virus.

2. Use according to claim 1 wherein said inactivated virus is administered orally.

3. Use according to claim 1 wherein said inactivated virus is administered intravenously.

4. Use according to any of claims 1 to 3 wherein said dosage is greater than about 10^{11} viral particles/kg patient body weight.

5. Use according to any of claims 1 to 4 wherein said inactivated virus is a whole virus.

6. Use according to any of claims 1 to 4 wherein said inactivated virus is a replication-defective virus.

7. Use according to claim 6 wherein said inactivated virus is selected from the group consisting of replication-defective retrovirus, replication-defective vaccinia virus, and replication-defective canarypox virus.

8. Use according to any of claims 1 to 7 wherein said inactivated virus comprises said recombinant virus carrying a transgene, previously subjected to inactivation.

9. Use according to any of claims 1 to 7 wherein said inactivated virus has identical viral sequence to said recombinant virus carrying a transgene, but said inactivated virus does not carry said transgene.

10. Use according to any of claims 1 to 7 wherein said inactivated virus and said live virus have antigenically related capsid proteins or express antigenically related viral proteins.

11. Use according to any of claims 1 to 7 wherein said inactivated virus is administered between about 6 hours to about 28 days before administration of said live virus.

12. Use according to any of claims 1 to 7 wherein said inactivated virus is re-administered before second and subsequent administrations of said live virus.

13. Use according to any of claims 1 to 7 wherein said inactivated virus is prepared by a method selected from the group consisting of thermal inactivation, physical inactivation, chemical inactivation and combinations thereof.

14. Use according to any of claims 1 to 7 wherein said inactivated virus and said live virus have different viral genes but carry substantially identical transgene sequences and express *in vivo* substantially identical transgene products.

15. A method for tolerizing a mammalian subject to administration of a live recombinant virus carrying a transgene for delivery to a cell of said subject comprising administering to said subject a high dosage of an inactivated virus prior to administration of said live virus.

1 / 3

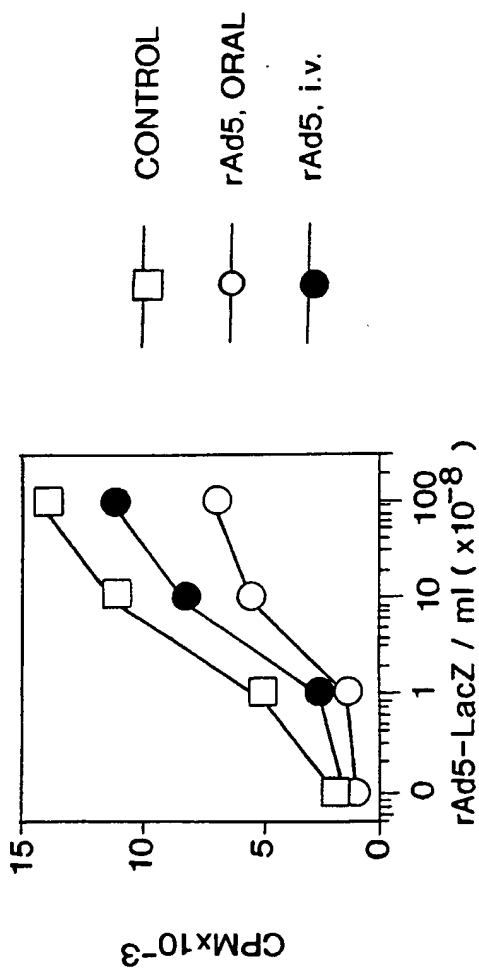


FIG. 1A

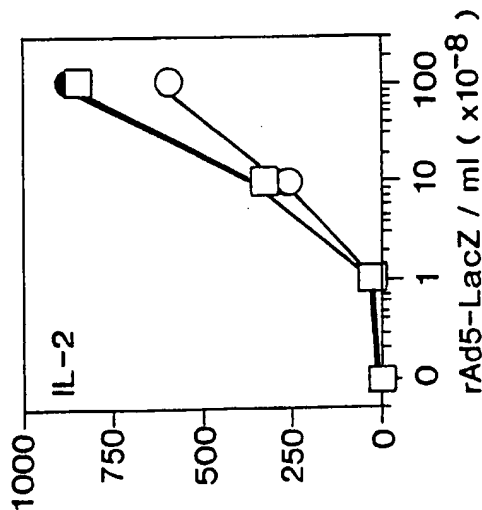


FIG. 1B

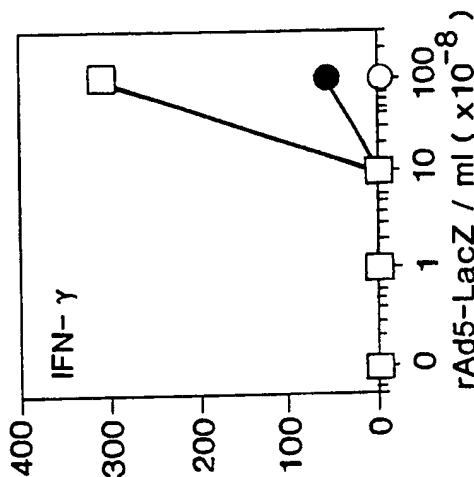


FIG. 1C

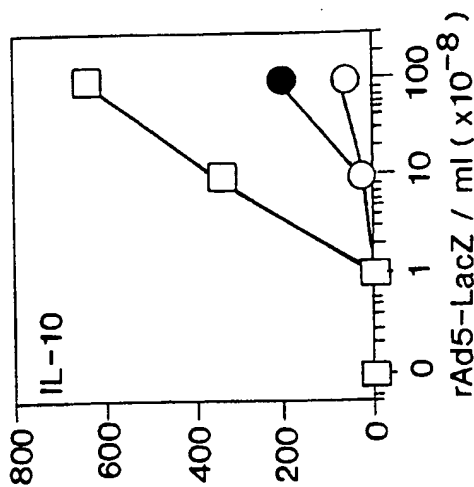


FIG. 1D

2 / 3

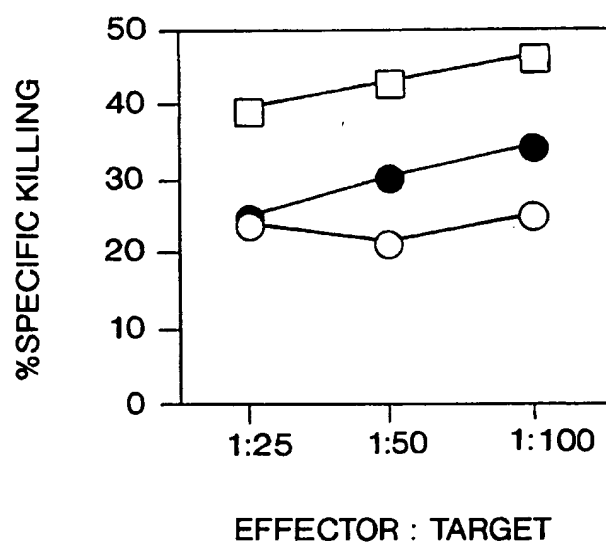
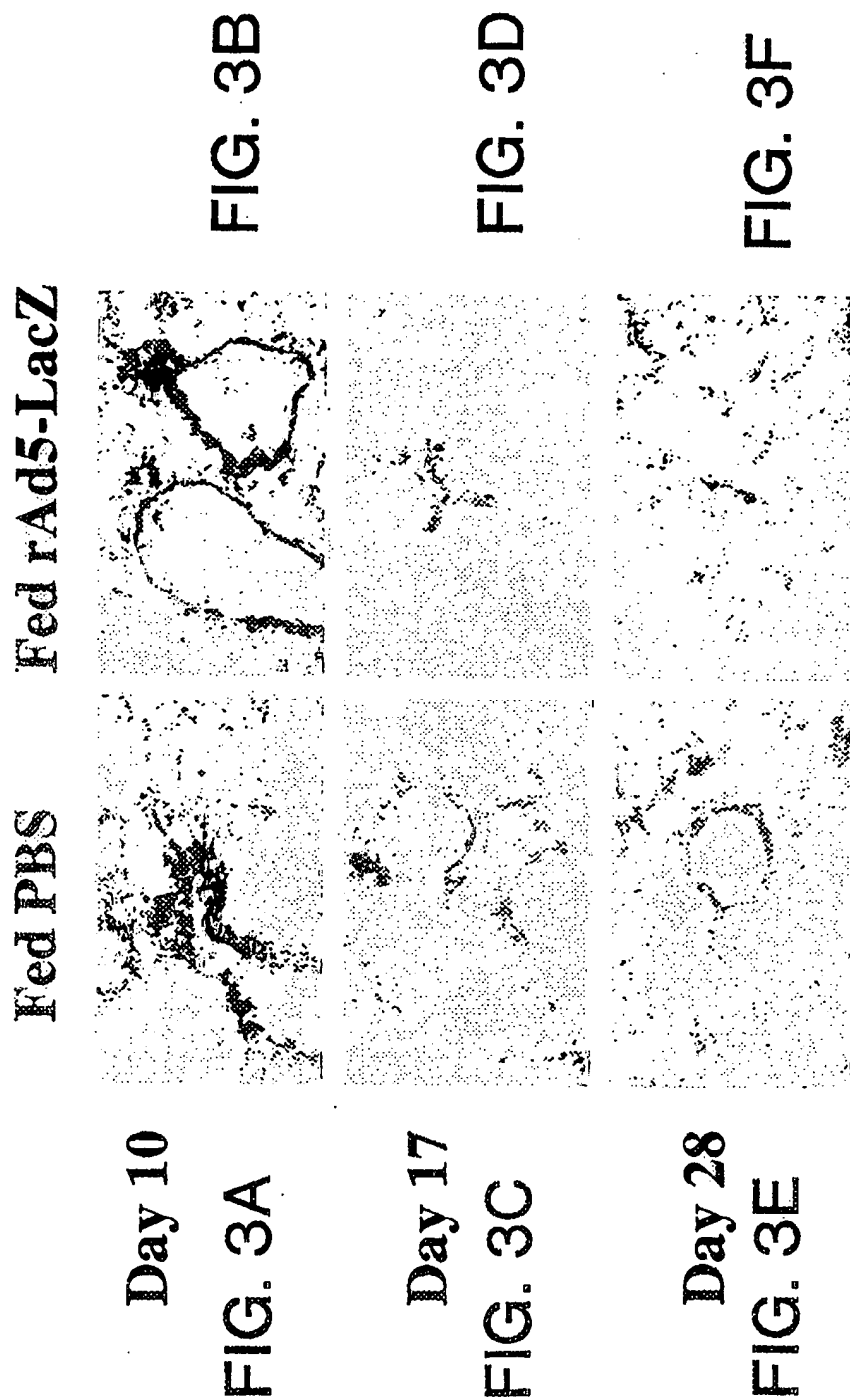


FIG. 2



INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/15690

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 14874 A (UNIV MANITOBA ;SEHON ALEC (CA); LANG GLEN M (CA)) 23 May 1996 see page 6, line 14 - page 7, line 29 ---	
A	WO 96 12406 A (GENETIC THERAPY INC ;TRAPNELL BRUCE C (US); YEI SOONPIN (US); MCCL) 2 May 1996 cited in the application see page 6, paragraph 6 - page 7, paragraph 3 see page 12, paragraph 3 - page 16, paragraph 2 ---	
A	WO 91 12816 A (AUTOIMMUNE INC ;BRIGHAM & WOMENS HOSPITAL (US)) 5 September 1991 see page 4, line 30 - page 5, line 19 --- -/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *Z* document member of the same patent family

Date of the actual completion of the international search

9 December 1997

Date of mailing of the international search report

09.01.98

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Sitch, W

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/15690

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>DEMURU ET AL: "IMMUNE TOLERANCE TO A DEFINED HETEROLOGOUS ANTIGEN AFTER INTRASPLENIC HEPATOCYTE TRANSPLANTATION: IMPLICATIONS FOR GENE THERAPY" FASEB JOURNAL, vol. 6, 1992, pages 2836-2842, XP002049566 see page 2836 see abstract</p>	
A	<p>--- KOZARSKY ET AL: "IN VIVO CORRECTION OF LOW DENSITY LIPPROTEIN RECEPTOR DEFICIENCY IN THE WATANABE HERITABLE HYPERLIPIDEMIC RABBIT WITH RECOMBINANT ADENOVIRUSES" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, 1994, pages 13695-13702, XP002049567 cited in the application see page 13695 see abstract</p>	
A	<p>--- YANG ET AL: "CELLULAR AND HUMORAL IMMUNE RESPONSES TO VIRAL ANTIGENS CREATE BARRIERS TO LUNG-DIRECTED GENE THERAPY WITH RECOMBINANT ADENOVIRUSES" JOURNAL OF VIROLOGY, vol. 69, 1995, pages 2004-2015, XP002049568 cited in the application see page 2004 see abstract see page 2014, paragraph 6 -----</p>	

INTERNATIONAL SEARCH REPORT

Int. application No.
PCT/US 97/15690

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/SA/ 210

Remark : Although claim 15 is directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/15690

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9614874 A	23-05-96	AU 3813395 A	06-06-96
WO 9612406 A	02-05-96	EP 0804076 A	05-11-97
WO 9112816 A	05-09-91	AT 157257 T	15-09-97
		AU 651097 B	14-07-94
		AU 7578991 A	18-09-91
		CA 2077340 A	03-09-91
		DE 69127470 D	02-10-97
		EP 0594607 A	04-05-94
		EP 0782859 A	09-07-97
		HU 61896 A	29-03-93
		HU 9500088 A	28-06-95
		IL 97446 A	31-03-96